Assisted Reproductive Technology

Accomplishments and New Horizons

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Spermatogenesis in vitro in mammals

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Introduction

Since the introduction of the first tissue cultures at the beginning of the twentieth century (embryonic fragments of nervous tissues: Harrison, 1907; fragments of chicken heart: Carrel, 1912), we have been able to study the behavior of animal cells without the complexities and systemic variations that arise in living animals.

Tissue culture has also been used to study testicular function without interference from hormones and to study cultures of isolated cells without the local controls imposed by the neighboring cells with which they normally communicate in situ. This technique was first described by Champy (1920), who maintained the testes on a "culture medium" constituted by a plasma clot as originally proposed by Carrel (1912). Since then, numerous attempts have been made to culture testes, testicular fragments, segments of seminiferous tubules, and dispersed heterogeneous or homogeneous populations of testicular cells, with varying degrees of success. Only a few reviews have been written on the subject (Wolff and Haffen, 1965; Kierszenbaum, 1994). However, because of the renewed interest in the culture of primary testicular explants or cells in mice, rats, and humans, we will review and analyze the data obtained in their historical and biological contexts. In view of the extremely complex cellular organization of the testis and the highly sophisticated mechanisms that control spermatogenesis, this review will begin with a brief overview of the anatomy of the testis, spermatogenesis, and the hormonal and paracrine control of this process.

Anatomy of the testis, spermatogenesis and its control

Although the genetic determination of sex of mammals occurs at fecondation, both sexes are morphologically identical until 12 days postcoitum (dpc) in the mouse, 13.5 dpc in the rat, and until week 7 of gestation in humans. The first recognizable event is the appearance of Sertoli cells in the gonads just after the expression of the sex-determining gene (*SRY*; Magre and Jost, 1980; Orth, 1993; Schmahl et al., 2000). Shortly after entering the genital ridge, the primordial germ cells (PGC), which migrate from the mesoderm, take a male pathway if their genotype is male and if the gonad has a male phenotype (McLaren, 1983). Fetal Sertoli cells aggregate to form the seminiferous cord and eventually enclose the PGC, which are then termed gonocytes. The developing seminiferous cords are rapidly surrounded by a basal lamina in the rat. At about the same time, flat mesenchymal cells become associated with the basal portions of Sertoli cells (Magre and Jost, 1980). In humans, the seminiferous cords become well organized by the end of week 9, and well-organized surrounding peritubular cells are visible by week 18 (Wartenberg, 1989; Orth, 1993). Recognizable Leydig cells appear in the interstitium of the fetal testis after the formation of the seminiferous cords: one to two days postpartum

(dpp) in the rat and about 1 week postpartum in humans (Orth, 1993).

Differentiation and organization of the testis

In the adult testis, the seminiferous tubules contain germ cells in various phases of development and nonproliferating Sertoli cells, which are surrounded by peritubular cells. Germ cells, which are continuously renewed, and Sertoli cells, which secrete several hormones and cease to divide during pubertal development, form the seminiferous epithelium. The primary function of the seminiferous tubules is the production of spermatozoa. The convoluted seminiferous tubules of the differentiated gonad are embedded in a connective tissue matrix, called the interstitium, which contains interspersed blood and lymphatic vessels, nerves, fibroblastic cells, macrophages, lymphocytes, rare mast cells, and the Leydig cells. The primary function of the Leydig cells is to produce testosterone.

Spermatogenesis

The complexity of the lining of the seminiferous epithelium is unique (Fawcett, 1975) and, therefore, the complexity of the communication network of cellular activities in the seminiferous tubules is also unique (Jégou, 1993; Gnessi et al., 1997; Jégou et al., 1999). This anatomical and functional communication network is established from the very beginning of the seminiferous cord formation (Pelliniemi et al., 1984; Byskov, 1986; Jégou, 1993; Orth, 1993). The period during which PGC and gonocytes divide is known as prespermatogenesis (Hilscher et al., 1974), whereas the formation of spermatozoa from the most immature germ cells in the postpartum period is called spermatogenesis. Spermatogenesis is classically divided into three phases. In the first, the proliferative or mitotic phase, primitive germ cells, or spermatogonia, replenish their stock and undergo a series of mitotic divisions. Spermatogonia arise from the differentiation of gonocytes after birth (3 dpp in the rat; McGuinness and Orth, 1992). In the second, meiotic phase, the spermatocytes undergo two consecutive divisions to produce the haploid spermatids. In the third, spermiogenic, phase or spermiogenesis, spermatids metamorphose into spermatozoa. Before any round of spermatogenesis is completed, new spermatogonial divisions are initiated; thus these three phases of spermatogenesis occur simultaneously in the tubules, permitting the production of 100 to 200 million spermatozoa per day in men, and up to several billion according to the mammalian species considered. The overall duration of spermatogenesis is approximately 35 days in the mouse, 50 days in the rat, and 70 days in humans (Courot et al., 1970).

In any given segment of the tubule, several germ cell generations develop simultaneously between the base and the apex of the epithelium. These generations are in close contact with Sertoli cells. The evolution of each generation of germ cell is strictly synchronized. This leads to the formation of defined cell associations. Such associations are known as stages of the seminiferous cycle. The succession of a complete series of stages in a given area of the tubule constitutes the seminiferous epithelial cycle. The duration of the seminiferous epithelial cycle is approximately 9 days in the mouse, 13 days in the rat, and 16 days in humans (Courot et al., 1970). At least four consecutive cycles are required for the complete evolution of a spermatogenic series from the stem spermatogonium to the mature sperm. Fourteen stages have been characterized in the rat (I–XIV; Leblond and Clermont, 1952). Six stages have been identified in humans (I–VI; Clermont, 1963). Unlike most other mammals, including the rat, multiple stages of the cycle can be seen in a single tubular cross-section in humans and a few other primates (Clermont, 1963; Leidl, 1968; Chowdhury and Marshall, 1980). This may be because in most mammalian species the different stages of the seminiferous epithelium cycle follow one another linearly, whereas in a few primates, including humans, a helical pattern may exist (Schulze and Salzbrunn, 1992), thus explaining the mosaic pattern of distribution of the stages of the cycle on each tubular cross-section. However, Johnson (1994) has challenged the existence of complete helically organized spermatogenic waves in humans.

Sertoli cells and the spermatogenic process

Sertoli cells are large cells extending from the innermost layer of the basement membrane that lines the seminiferous tubules towards the lumen. Their cytoplasmic processes envelop the associated germ cells. Because of their position in the seminiferous epithelium, Sertoli cells have the unique ability to communicate with all germ cell generations and with the myoid cells. They can also communicate with the paracrine and endocrine signals originating from both the interstitium and the bloodstream via their bases. Androgen production and Sertoli cell function are controlled by the pituitary hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH), respectively. Testosterone produced by the action of LH or FSH on the Leydig cells is required for quantitatively and qualitatively normal spermatogenesis (Sharpe, 1994; Weinbauer et al., 2000). The action of these hormones on spermatogenesis is mediated by the Sertoli cells, which express testosterone and FSH receptors, whereas germ cells do not (Jégou, 1992; Weinbauer et al., 2000).

In addition to transducing the hormonal signals, the position of Sertoli cells within the seminiferous tubules allows them to play a key role in the paracrine control of spermatogenesis. Sertoli cells supply each individual germ cell generation, at each stage of testicular development, with the factors needed for their division, differentiation, and metabolism. It is believed that they also assist germ cells in synchronizing their development in the stages of the epithelium cycle, in the transversal axis of the tubule. Furthermore, Sertoli cells and the bridges that interconnect each germ cell generation are key features in the establishment of germ cell synchrony and probably contribute to the maintenance of the wave of spermatogenesis along the longitudinal axis.

On an anatomical basis, Sertoli cells and germ cells communicate via a unique set of structural devices (Russell, 1984; Jégou et al., 1992; Jégou, 1993). During the postnatal development of the testis, the establishment of inter-Sertoli cell tight junctions in the basolateral portion of the cell indicates that Sertoli cells have matured and, therefore, that the meiotic phase of spermatogenesis can proceed. These tight junctions are the major tubular constituent of the blood–testis barrier, which prevents a number of substances present in the testicular blood and lymph vessels from penetrating the seminiferous epithelium. The Sertoli cell barrier divides the seminiferous epithelium into a basal compartment, containing spermatogonia and early primary spermatocytes, and an adluminal compartment, which contains other differentiated primary spermatocytes, secondary spermatocytes, and the various stages of haploid spermatids. The Sertoli cell barrier creates a unique microenvironment that is essential for normal meiosis and spermiogenesis.

Most, if not all, Sertoli cell products assist germ cells through the three phases of spermatogenesis. The tubule fluid is essential for the nutrition of germ cells and for the transport of signals in the transversal axis of the seminiferous epithelium and along the tubule. It is also required for the transport of spermatozoa along the tubules, from the tubules to the rete testis and subsequently to the epididymis (Jégou, 1992).

Although germ cells are under the control of Sertoli cells, they can exert feedback actions on them (Jégou, 1993; Griswold, 1995; Gnessi et al., 1997; Jégou et al., 1999).

The requirement of in vitro spermatogenesis studies

The extremely complex in vivo structural organization of the mammalian testis creates particular difficulties for studying their organization, function, and regulation. This has provided the motivation for developing in vitro systems of spermatogenesis since testicular tissues were first cultured by Champy (1920). The following reasons justify the development of these systems: (i) the necessity to design experiments to determine the role of hormones in spermatogenesis; (ii) the need to study the role of Sertoli cells in the control of spermatogenesis and of putative local regulatory factors on germ cell

division, differentiation, and metabolism; (iii) the necessity to study the molecular and cellular mechanisms characterizing and/or controlling each of the different phases of spermatogenesis in normal or pathophysiological contexts; (iv) the generation of germ cells that can repopulate the seminiferous tubules of recipient animals after microinjection; and (v) the production of germ cells and spermatozoa from testicular biopsies from men with spermatogenic arrest to be used for intracytoplasmic sperm injection (ICSI) or elongated spermatid injection (ELSI).

With one, or several, of these goals, the following activities have been developed in this domain: (i) the culture of whole testes, of testicular explants, or of segments of seminiferous tubules; (ii) the culture of mixtures of dissociated testicular cells either for direct use or for transplantation by microinjection into the aspermatogenic seminiferous tubules of recipient animals; (iii) the culture of isolated germ cells purified at particular phases of their development and of spermatogenesis; (iv) the co-culture of purified or unpurified Sertoli cells (primary cultures or cell lines) and germ cells; and (v) the design and culture of immortalized germ cell lines.

In the next section we shall endeavor to describe and to analyze critically the various in vitro systems of spermatogenesis that have been developed and the results generated by these systems.

The culture of testicular tissues

Fetal testis fragments

The differentiation of testes from undifferentiated mouse or rat primordia during in vitro culture has been reported since 1952 (Wolff, 1952; Asayama and Furusawa, 1960, 1961; Byskov and Saxen, 1976; Taketo and Koide, 1981; Agelopoulou et al., 1984; McGuinness and Orth, 1992; Buehr et al., 1993; Olaso et al., 1998). Several key observations have been made using these in vitro systems, particularly in the mouse and rat: (i) cellular mesonephric contribution is required for the establishment of seminiferous cords (Taketo and Koide, 1981; Buehr et al., 1993); (ii) the proliferation and relocation of gonocytes both begin and continue in culture (McGuinness and Orth, 1992); (iii) Sertoli cells produce bioactive antimüllerian hormone, which induces regression of Müller's duct and is required for germ cell development in neonatal mouse testes (Zhou et al., 1993); (iv) gonocytes spontaneously reenter meiosis in cultured neonatal testes (McGuinness and Orth, 1992); (v) transforming growth factor (TGF) β_1 and β_2 directly increase apoptosis in gonocytes, without changing their mitotic activity during the developmental phases of proliferation (Olaso et al., 1998); and (vi) fetal testicular -β_1 is stimulated significantly by FSH and even more by a combination of LH and FSH (Gautier et al., 1997).

Cell reaggregation experiments in mice showed that the histogenic behavior of germ cells is markedly modified with age. In fact, most germ cells that had been dissociated from fetal testes at 12.5 dpc could be reincorporated into the seminiferous cords, although those dissociated at 14.5 dpc could not. This may be a consequence of changes in Sertoli cell surface properties, which are crucial for their binding to gonocytes when the gonocytes enter their mitotic resting stage (Escalante-Alcalde and Merchant-Larios, 1992).

Finally, only a few spermatocytes developed in vitro when newborn or rats aged one to three days postpartum (dpp) were used (Steinberger, 1967, 1975). This suggests that the differentiation of gonocytes to primitive type spermatogonia is sensitive to culture conditions. This was confirmed by Gelly et al. (1984), who found no differentiation of the germinal elements of the seminiferous tubules from 5 dpp rats after culture for 4 to 12 days.

Postnatal crude testis fragments

In early cultures of postnatal testes or testicular fragments (Champy, 1920; Martinovitch, 1937; Gaillard and Varossieau, 1938, 1940), a plasma clot was used to provide support and a source of nutrients. Therefore, it was not clear whether the spermatogenic cells had developed in vitro or whether they

already existed at the onset of culture. Adult testes degenerated on the first day of culture, whereas prepubertal testes survived for eight to nine days (Gaillard and Varossieau, 1940). However, these early attempts were greatly hampered by the lack of objective methods for following cell differentiation.

Trowell (1959) introduced a method in which small tissue fragments were cultured in a chemically defined medium on stainless-steel grids covered with lens paper or with a thin sheet of agar at the liquid–gas interphase. This method was used for long-term cultures of testes from several mammalian species by Steinberger and Steinberger (Steinberger et al., 1964a,b, 1970; Steinberger and Steinberger, 1967; Steinberger, 1967). Tubular architecture, Sertoli cells, and primitive type A spermatogonia could be maintained for six to eight months. Rat spermatocytes survived for three to four weeks, but spermatids only survived for a few days. In an attempt to follow in vitro differentiation, rat preleptotene spermatocytes, which are the most advanced spermatogenic cells replicating DNA, were labeled with [^{3}H]-thymidine and their development was subsequently followed by autoradiography (Steinberger and Steinberger, 1965, 1967, 1970). These cells developed to a late prophase of meiosis, but meiotic divisions and spermiogenesis were never observed (Steinberger and Steinberger, 1970, 1971). Degenerative changes were more prominent in testes from adult animals than in those from prepubertal ones (Steinberger, 1967; Steinberger et al., 1970). This is obviously because of the rapid degeneration of postmeiotic cells in mature testes. Spermatogenic capacity was maintained in vitro for at least seven weeks, because spermatogenesis was completed within 8 to 10 weeks after the cultured fragments had been reimplanted into the testes of adult homologous hosts (Steinberger et al., 1970). Supplementation of the growth medium with vitamins A, C, and E, or glutamine enabled rat primitive type A spermatogonia from prepubertal testes to develop to the pachytene stage of meiosis, but new cell generations did not appear after the first cycle of development (Steinberger and Steinberger, 1966a). Gonadotropins and testosterone did not improve germ cell development (Steinberger et al., 1964c, 1970; Steinberger and Steinberger, 1967).

Spermatogonia from mouse cryptorchid testes could reinitiate meiosis in vitro (Aizawa and Nishimune, 1979). FSH in combination with insulin and transferrin may be able to promote the mitotic activity of type A spermatogonia, although insulin, transferrin, testosterone, dihydrotestosterone, triiodothyronine, dibutyryl 3′,5′-cyclic adenosine monophosphate (cAMP), human chorionic gonadotropin, LH, and FSH alone showed no stimulatory effect (Haneji and Nishimune, 1982). Furthermore, retinoids activated cell division in type A spermatogonia and induced their differentiation in vitro (Haneji et al., 1982, 1983a, 1986) whereas FSH had synergistic effects on in vitro spermatogenic cell differentiation when cells were also treated with retinoids or Pedersen type III fetuin (Haneji et al., 1983b, 1984). However, spermatogenic cells that differentiated from cryptorchid adult testis died in the pachytene stage of meiosis (Haneji et al., 1984). On the basis of the numerous studies mentioned above, it has been generally believed that mammalian spermatogenesis cannot be maintained in organ culture beyond the prophase of meiosis (Steinberger, 1975; Setchell, 1978).

However, when rat seminiferous tubular segments from the stages immediately preceding the meiotic divisions were cultured, despite the rapid degeneration of a very large fraction of germ cells, completion of the divisions and early spermiogenesis could be shown (Parvinen et al., 1983). The extent of differentiation was the same with or without the addition of growth factors or hormones (testosterone, FSH). This led to a series of studies by Toppari and collaborators (Toppari et al., 1985, 1986a,b), in which segments of adult rat seminiferous tubules from defined stages of the epithelial cycle were cultured in an attempt to trace the differentiation of spermatogenic cells. The desired stages were isolated by transillumination-assisted microdissection combined with accurate identification of the stages by phase contrast microscopy. Cultures were started at stages II–III, VI, VIII, and XII–XIII of the epithelial cycle and continued for one to seven days in a chemically defined medium. It

was found that premeiotic and meiotic phases of spermatogenesis, and the Golgi and cap phases of spermiogenesis, proceeded according to the same time schedule in vitro and in vivo, and the cells differentiated in vitro seemed to be morphologically and functionally normal. However, numerous spermatogenic cells degenerated in culture. The acrosome and maturation phases of spermiogenesis did not occur in vitro. Complete spermiogenesis and better survival of spermatogenic cells in vitro are the main challenges for the development of culture conditions in future.

Spermatogenic cells could be quantified by DNA flow cytometry (Toppari et al., 1985, 1986a). The number of cells obtained at defined stages was consistent with the results of previous morphometric assessments. Different stages had typical DNA histograms, characterized by the location of the hypo–haploid peak caused by the spermatid maturation phase, and by the relative proportions of cells in each DNA class. Secretion of plasminogen activator (PA) was used as a marker of cyclic Sertoli cell function and as an index of local hormone action in seminiferous tubules. The cyclic PA secretion was partially maintained in vitro. FSH was found to stimulate PA activity in stage VIII, but not in stage VI. A combination of FSH, insulin, testosterone, and retinoic acid stimulated both of these stages, whereas testosterone alone had no effect. The results indicated that the effect of FSH was highly stage specific. The seminiferous tubules mainly secreted urokinase-type PA, but tissue-type PA was occasionally found in the culture medium of stage VIII after stimulation by FSH or a combination of FSH, insulin, testosterone, and retinoic acid.

Use of this approach to culture seminiferous tubule fragments at defined stages of the cycle led to the development of a new method to test male germ cell mutagenicity, based on the induction of micronuclei during meiotic divisions in vitro. A model mutagen, adriamycin, caused a dose-dependent increase of micronucleus induction in vitro, at concentrations well below toxic levels (Toppari et al., 1986b). Mixed cultured segments of seminiferous tubules were used in toxicological studies (Allenby et al., 1991) collected at different stages of the cycle from rats that had been exposed to methylmethanesulfonate. Bentley and Working (1988) separated the tubule segments to show that this in vitro system increased the sensitivity of unscheduled DNA synthesis as a function of DNA damage in rat germ cells at different stages of maturity.

Recently, Durand and coworkers reinitiated the use of rat tubular segment cultures for studying spermatogenesis in vitro (Hue et al., 1998; Staub et al., 2000). They used unselected segments, premeiotic rat testes, and a bicameral culture system with a chemically defined medium supplemented with vitamins and hormones, including FSH and testosterone. Furthermore, an unprecedented number of criteria were used to monitor germ cell differentiation: (i) cytological light and electron microscopy immunocytochemical observations; (ii) measurement of genes specifically expressed in pachytene spermatocytes (phosphoprotein p19) and in early spermatids (transition proteins 1 and 2); (iii) ploidy analyses by image analysis; and (iv) the study of the fate of bromodeoxyuridine (BrdU)-labeled leptotene spermatocytes. It was found that, although massive germ cell death was encountered under the relatively long-term culture conditions used (up to three weeks for tubule fragments), a number of pachytene spermatocytes were unequivocally able to differentiate into secondary spermatocytes and then into early spermatids (Hue et al., 1998; Staub et al., 2000). The rate of differentiation of the germ cells observed in these in vitro studies was consistent with the in vivo situation as previously observed by Parvinen et al. (1983) and Toppari et al. (1985).

Most attempts to establish and to study spermatogenesis in vitro were carried out on animals, particularly on rats. As little fresh material is available, few contemporary studies have been carried out on human testes or human testicular fragments. Ghatnekar et al. (1974) used an autoradiographic approach to show that the first meiotic division was completed after 14 days in an organ culture of three human testes at 36°C in a medium supplemented with fetal calf serum (FCS), deproteinized coconut

milk, and gonadotropic hormones. Curtis (1981) performed similar cultures without hormones and coconut milk and found labeled diakinetic figures after 10 to 14 days in culture, but did not observe labeled division figures. Heller and Clermont (1963) found that spermatogonia and preleptotene spermatocytes are the only cell types that incorporate [^{3}H]-thymidine in adult human testis, and that preleptotene spermatocytes differentiate to early to mid pachytene spermatocytes after 14 days in vivo. Therefore, it is unlikely that these cells could have reached this stage in that time in vitro, particularly as the workers used an unphysiological temperature of 36°C (VanDemark and Free, 1970). Seidl and Holstein (1990) cultured human seminiferous tubules that had been mechanically isolated and had the cut edges sealed, at the more favorable temperature of 34.5°C. In this study, the beneficial influences of FCS, of nerve growth factor (NGF), and of various additional supplements were assessed. The evaluation of the germ cell populations was monitored by [^{3}H]-thymidine labeling combined with light and electron microscopy. It was shown that, in absence of medium supplements, but in presence of 20% FCS, all germ cell types degenerated within three weeks, except dark and pale type A spermatogonia. In the chemically defined medium, only 33.3% of the cross-sections of the tubules cultured for five days without FSH contained labeled germ cells, whereas 83.3% of those from tubules exposed to FCS showed mitotic activity. In these tubules, FCS doubled the number of [^{3}H]-thymidine-labeled germ cells per cross-section. Furthermore, NGF together with FCS had the most beneficial effect on the maintenance of the seminiferous epithelium. NGF action directly maintained a better basal lamina and Sertoli cells integrity.

However, the most spectacular results of human in vitro spermatogenesis were published by Tesarik and coworkers, who used human testicular fragments (Tesarik et al., 1998a,b, 1999, 2000a–c). They reported that in high concentrations of FSH both meiotic and postmeiotic maturation can occur in germ cells collected from testicular biopsies from men with normal spermatogenesis or with maturation arrest after only 24 to 48 hours of culture (it is known that the normal duration of spermatogenesis in humans is approximately 70 days, and that the meiotic prophase and spermiogenesis each last more than three weeks). They also reported that babies have been born following the microinjection of spermatids obtained by in vitro maturation of germ cells from azoospermic patients affected by a spermatogenic maturation arrest (Tesarik et al., 1999). However, the protocols used by this group lacked the prerequisites for an unequivocal demonstration of spermatogenesis in vitro. These studies used insensitive or inappropriate techniques to assess cell viablility, to count cells, and to monitor cell differentiation. In the absence of appropriate techniques, we cannot determine whether germ cells really can mature at such an extraordinary velocity (Tesarik et al., 1998a,b). Furthermore, Tesarik et al. (2000b) claimed that other studies on the rat (Parvinen et al., 1983; Le Magueresse-Battistoni et al., 1991; Weiss et al., 1997; Hue et al., 1998) and on humans (Tres et al., 1989) have also shown that meiotic and postmeiotic differentiation events occur more rapidly in vitro than in vivo, although this is not the case (Durand et al., 2001; Jégou et al., 2001). This does not clarify the actual contribution of this group to the development of a culture system to perform human spermatogenesis in vitro.

The culture of testicular cells

Until the recent advances in tissue culture, studies on testicular function had progressively stopped using cultures of testicular fragments and dissected segments of seminiferous tubules, and had concentrated on the development of more sophisticated culture systems, in which Sertoli cells and germ cells were either carefully dispersed in more or less small aggregates or were isolated and purified before being cultured or co-cultured. An attempt was made to develop the tools required to enable better monitoring of the maturation of germ cells and for an analytical study of the interactions between Sertoli cells and germ cells.

Culture of Sertoli cells and of small aggregates of germ cells

Several studies aimed to enrich cell preparations of Sertoli cells and small aggregates of germ cells without breaking their interconnecting junctions and intercellular bridges. Kierszenbaum (1994) reported that the different factors required for successful preparation of Sertoli cell–germ cell co-cultures are: (i) careful enzymatic dissociation to alter the structural interactions between Sertoli cells and germ cells as little as possible; (ii) plating of the aggregates at maximal cell density to keep Sertoli cells in a contact-inhibited state; (iii) the use of a chemically defined medium (serum-free) supplemented with hormones, such as testosterone, dihydrotestosterone, growth hormone, FSH, and growth factors; and (iv) frequent changes of culture medium. Tres and Kierszenbaum (1983) used such a system on prepubertal and pubertal rat testes and showed that in cell reaggregation experiments: (i) germ cells reassociate preferentially with Sertoli cells; (ii) polygonal spermatogonia form long, branched chains of interconnected cells; (iii) that [^{3}H]-thymidine-labeled spermatogonia and preleptotene spermatocytes connected by cytoplasmic bridges have a synchronous S phase; and (iv) labeled preleptotene spermatocytes can progress throughout meiotic prophase stages. They also showed that round spermatids can grow flagella in vitro, but that, although the axoneme of these cells display a typical wave-like motion, the flagella lack outer dense fibers (Tres et al., 1991). The time course of the replacement of the testis-specific histone variants TH2B and H1t in primary spermatocytes in vitro is consistent with that observed in vivo (Smith et al., 1992).

In 1985, Hadley et al. used a reconstituted basement membrane gel to show that some spermatogonia can differentiate to pachytene spermatocytes in a Sertoli cell culture.

Culture of dispersed testicular cells

Primordial germ cells PGC and gonocytes

A few methods have recently been developed that allow the isolation and purification of migratory and postmigratory mouse and rat PGC. Van Dissel-Emiliani et al. (1989) used velocity sedimentation at unit gravity to isolate embryonic gonocytes (70–75% enrichment) from 18 dpc rats. A few years later, De Felici and Pesce (1995a) proposed the use of a PGC-specific TG-1 antibody in combination with immunoaffinity adhesion to plastic plates coated with an anti-mouse IgM secondary antibody for the isolation of migratory PGC from 9.5 to 11.5 dpc mouse embryos and achieved reasonably pure yields. De Felici and Pesce further refined this technique so that the cell sorter MiniMACS magnetic separation system could purify PGC from 10.5 to 13.5 dpc mouse embryos (De Felici and Pesce, 1995b). More recently, van den Ham et al. (1997) adapted a direct immunoseparation technique for the isolation of gonocytes from 18 and 20 dpc rat embryos. This technique used magnetic beads coated with a rat anti-mouse IgM and a monoclonal antibody 4B6.3E10, which specifically reacted with a differentiation antigen on the fetal germ cells.

In a pioneer study, De Felici and McLaren (1983) followed the survival of PGC isolated from 11.5 to 16.5 dpc mouse embryos in single culture using Petri dishes, microtiter plates, or drops under oil. They showed that PGC from embryos at 11.5 and 12.5 dpc could not survive at 37 °C in any of the culture systems used, but that they could survive at 30 °C for at least a week. Interestingly, PGC from 13.5 dpc embryos onwards survived at 37 °C for several days. PGC did not enter meiosis in any culture conditions, but they continued to undergo mitotic proliferation. Several attempts were also made to study the survival and proliferation of freshly isolated PGC/gonocytes in vitro (mouse: De Felici and Dolci, 1989, 1991; Matsui et al., 1991; rat: Li et al., 1997; van Dissel-Emiliani et al., 1993). These attempts yielded a couple of significant observations. The addition of extracellular matrix components (laminin, fibronectin, type I collagen) scarcely improves PGC survival (De Felici and Dolci, 1989), and PGC and gonocytes cannot survive for more than 2 days in vitro (De Felici and Dolci, 1991; Matsui et al., 1991; van Dissel-Emiliani et al., 1993; Li et al., 1997). Mitosis did not resume when gonocytes are cultivated alone (Li et

al., 1997). Interestingly, the effects of various compounds on the survival and proliferation of PGC/gonocytes in single culture have been studied (for reviews, see De Felici, 2000; Olaso and Habert, 2000). For example, it has been proposed that stem cell factor and leukemia inhibitory factor (LIF) are survival anti-apoptotic factors for PGC (De Felici and Dolci, 1991; Dolci et al., 1993; De Felici, 2000), whereas TGF-β_1 is required for PGC survival (De Felici and Pesce, 1994a), and that platelet-derived growth factor and estradiol activate gonocyte proliferation (Li et al., 1997). Furthermore, cAMP, pituitary adenylyl cyclase activating polypeptides (PACAPs), and retinoic acid appear to be positive regulators of PGC proliferation (De Felici, 2000). Finally, Di Carlo and De Felici (2000) demonstrated that the formation of germ cell aggregates, which occurs rapidly in culture when dispersed germ cell populations are released from embryonic gonads, and their development are mediated by E-cadherin, depending on the sex of the germ cells.

These studies clearly show that PGC or gonocytes require the environment provided by somatic cells to survive and to differentiate correctly. This highlights the in vivo finding that PGC which do not enter the cords during early development degenerate (Byskov, 1986).

Pubertal and postpubertal germ cells

Morphological analyses showed that primary spermatocytes and round spermatids survived for several days in culture of testicular cell suspensions (Steinberger and Steinberger, 1966b). However, no evidence of cell differentiation was obtained (Steinberger and Steinberger, 1970). Since the mid 1970s, it has been possible to prepare highly purified populations of mammalian germ cells at different stages of development (Grabske et al., 1975; Romrell et al., 1976; Bellvé et al., 1977a,b; Meistrich et al., 1981; Loir and Lanneau, 1982). However, the removal of cells from their normal physiological environment is intrinsically deleterious to all cell types, especially germ cells, which appear to be the least autonomous of all testicular cells in mammals. First, after birth, intact isolates of some germ cells classes cannot be obtained because of the structural intricacy of Sertoli cells. Second, in isolated culture, germ cell metabolism is greatly altered and the cells cannot survive for more than a few hours or days (Grootegoed et al., 1977, 1989; Jutte et al., 1981, 1982; Le Magueresse and Jégou, 1988; Matsui et al., 1991; Risley and Morse-Gaudio, 1992).

Despite their extreme fragility when isolated from culture, interesting information has been obtained on the behavior of isolated meiotic and postmeiotic germ cells. [^{3}H]-Leucine can be incorporated into isolated germ cells in vitro (Millette and Moulding, 1981), and isolated spermatocytes and early spermatids can synthesize various proteins during short culture periods (Gerton and Millette, 1986). However, it is not known whether these are "physiological" events or whether they reflect a stress response to culture conditions because the cells are known to die massively in these experimental conditions. Early mouse spermatids were able to generate flagella during a 24 hour culture period, but the individual germ cell differentiation was not carefully assessed in this study (Gerton and Millette, 1984). The optimum temperature for germ cell activity (particularly for spermatids) is 34 °C rather than 37 °C (Nakamura et al., 1978, 1984). Lactate and pyruvate are better substrates for spermatocytes and early spermatids in vitro than glucose, which cannot be metabolized (Nakamura et al., 1984). The presence of these two energy-providing substrates in Sertoli cell-conditioned media (Robinson and Fritz, 1981; Jutte et al., 1982; Le Gac et al., 1982, 1983; Mita et al., 1982) may explain the beneficial effect of these media, compared with the nonconditioned media, on pachytene spermatocytes and early spermatids in vitro (Grootegoed et al., 1982).

Recently, 50% of spermatogonia from an 80 dpp piglet were shown to be viable after 30 days when cultured in a potassium-rich medium (Dirami et al., 1999).

However, it is generally accepted that there is no primary culture system available that allows the long-term culture of purified spermatogonia (de Rooij and Russell, 2000) or of other categories of germ cell. Moreover, the annexin V technique showed that a

high percentage of rat spermatogonia or pachytene spermatocytes and early spermatids are already apoptotic upon isolation by gradient sedimentation at unit gravity or centrifugal elutriation (these procedures take 4 and 5 hours, respectively) (E. Guillaume, B. Jégou and C. Pineau, unpublished data).

Interestingly, it was recently shown that isolated pachytene spermatocytes from prepubertal rats that had been treated in culture with okadaic acid, a potent phosphatase inhibitor, reach metaphase I arrest within a few hours, unlike the similar process in vivo, which require several days (Handel et al., 1995; Tarsounas et al., 1999). Leptotene/zygotene spermatocytes cannot be activated in this way; therefore, okadaic acid may enable cells to bypass a sensor of meiotic progression that is specific to the pachytene stage, resulting in the rapid degradation of two meiotic-specific proteins SYN1/SCP1 and COR1/SCP3, subsequent desynapsis, and progression to metaphase I. If this system allows the rapid cytological and biochemical analyses of the meiotic events in a "fast forward" mode, as stated by Tarsounas et al. (1999), then it is noteworthy that the spermatocytes were mostly arrested at early metaphase I because of a lack of meiotic spindles. This demonstrates that the germ cells generated after "accelerated" spermatogenesis are very seriously degraded.

Human germ cells also die very quickly in vitro (within 24 to 72 hours) (Aslam and Fishel, 1998). However, flagellar growth occurs in approximately one fifth of human spermatids within 8 hours of culture. It is noteworthy that this growth was essentially unphysiological because it was not coupled to any of the other morphological changes that are normally seen during spermiogenesis in situ. Therefore, instead of being "an excellent way of identifying" viable spermatids, as stated by Aslam and Fishel (1998), this growth should probably be interpreted as an abnormal event connected to spermatid degeneration induced by cell isolation and culture. Consequently, the use of these cells for in vitro fertilization appears to be most risky.

Another group has claimed that extended maturation of human round spermatids in vitro can occur when these cells are co-cultured with Vero cells (Cremades et al., 1999). However, extremely low numbers of germ cells were cultured (2 to 37 depending on the experiment), and an objective method was not used (e.g., [^{3}H]-thymidine or BrdU labeling) to monitor individual cell differentiation. Furthermore, some of the changes occurring during cell degeneration (e.g., changes in the cell shape, flagellar extrusion, or growth) may have been confused with morphological aspects related to spermatid differentiation.

Johnson et al. (1999) performed a very complete comparative morphological study of human germ cells in vitro and in situ within tubules. This allowed the germ cells originating from human isolated seminiferous tubules in vitro to be objectively identified for the first time. The list of distinguishing characteristics of live human germ cells provided by these authors is a very precious tool, both for biologists who will plan future experiments on in vitro spermatogenesis and for technical staff in clinics for the selection of germ cells for in vitro fertilization.

All the in vitro experiments carried out with isolated purified mammalian germ cells in animals and men indicate that their differentiation is particularly dependent on the support of Sertoli cells; this is consistent with the findings of Wolff and Haffen in 1965 (initially quoted by Kierszenbaum, 1994).

The culture of spermatogonia represents a particularly important goal as, if it proves successful, it would allow the study of each step of spermatogenesis in vitro. Furthermore, it has been shown that a heterogeneous mouse testis cell suspension containing stem germ cells could be transplanted, by microinjection into the seminiferous tubules of sterile recipients and totally restore spermatogenesis, even after cryopreservation (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). This technique opens up enormous possibilities for the treatment of male sterility, for the protection of endangered species, for engineering genetically manipulated animals, and for determining the origin of arrested spermatogenesis in mutant or mutated experimental animals (for reviews, see Brinster and Nagano, 1998; Johnston et al., 2000; Schlatt et al., 2000).

Following the introduction of this technology, Brinster and coworkers have achieved a number of experimental developments. Crude mouse testicular cells, containing germ stem cells, could be cultured on a feeder layer of STO cells and in a medium containing 10% for four months. When transplanted to busulfan-treated recipient testes, these cells were able to regenerate full spermatogenesis. In contrast, no colonization occurred in absence of the feeder cells, clearly indicating that stem spermatogonia cannot survive or remain functional without somatic assistance (Shinohara et al., 2000). This group also have developed techniques that use a number of cell surface molecules to enrich fractions of mouse spermatogonial stem cells from cryptorchid testes (66-fold). These cells were fractionated by fluorescence-activating cell sorting analysis, based on their light scattering properties (Shinohara et al., 2000).

Several other techniques, which use either sedimentation velocity at unit gravity (Dym et al., 1995) or differential adhesion on plastic dishes coated with lectin (*Datura stramonium*) agglutinin and fractionation on discontinuous Percoll gradient (Morena et al., 1996,) have also been developed to isolate highly purified type A spermatogonia from prepubertal rat testis. Both techniques apparently allow a cell fraction containing up to 85% type A spermatogonia to be recovered. Van Pelt et al. (1996) also proposed a method to isolate synchronized type A spermatogonia from adult vitamin A-deficient rat testes, with a purity of 70–90%. More recently, von Schonfeldt et al. (1999) developed a magnetic cell sorting technique, based on an anti-c-Kit IgG, to enrich highly viable spermatogonia from testes of Djungarian hamster, mouse, and marmoset monkey.

As stated above, the poor viability of mammalian germ cells in culture does not allow their in vitro differentiation to be studied when they are isolated. Consequently, the establishment of immortalized mammalian germ cell lines, capable of in vitro differentiation, would be a major breakthrough. Hofmann and collaborators (1992, 1994, 1995) established an immortalized cell line (GC-1spg) by using the immortalizing properties of the simian virus 40 large tumor antigen (Hofmann et al., 1992). GC-1spg, characterized as a cell line at a stage between type B spermatogonia and primary spermatocyte, were unfortunately unable to differentiate further in vitro, even when co-cultured with immortalized Sertoli cells (Hofmann et al., 1992). More recently, Hofmann et al. (1994) cotransfected primary mouse testicular germ cells with the same simian virus 40 large T antigen gene and the gene coding for a temperature-sensitive (ts) mutant of p53 and established two new germ cell lines (GC-2spd(ts) and GC-3spc(ts)). They then used the property of p53 to abolish the proliferative function of the large T antigen when both molecules are expressed (Fukasawa et al., 1991) and to induce growth arrest at permissive temperatures in the studied cell lines. Both cell lines express the lactate dehydrogenase C_4 and cytochrome c_t isoforms, which are specific for meiotic and postmeiotic germ cells in vivo (Goldberg et al., 1977; Wheat et al., 1977). Moreover, proliferation rates for both cell lines were high at 39 °C, decreased at 37 °C, and were inhibited at 32 °C. Hofmann and coworkers (1994) claimed that at the permissive temperatures of 37 °C and 32 °C only the GC-2spd(ts) line could undergo meiosis and generate haploid cells with an acrosome granule and a flagellar axoneme. This confirms that these cells are early spermatids. However, it was recently reported that GC-2spd(ts) may have become less differentiated and may have lost their postmeiotic potentiality over time (Wolkowicz et al., 1996). Use of DNA flow cytometry to analyze GC-2spd(ts) during 10 days of culture did not detect a peak indicative of the presence of haploid chromosomes. The expression of mRNAs encoding stage-specific proteins (lactate dehydrogenase C4, acrosin, protamine-2, and SP-10) in GC-2spd(ts) was studied by Northern blotting and reverse transcriptase polymerase chain reaction (RT-PCR). These techniques did not reveal the presence of any of these transcripts (Wolkowicz et al., 1996). It is noteworthy that cell lines must be established without the irreversible loss of differentiated properties that is often associated with immortalization. Immortalization generally coincides with an extensive destabilization of the genome (Cerni et al.,

1987; Vogt et al., 1987). Therefore, the immortalization of germinal cells may be incompatible with their entrance into meiosis, for which genomic integrity appears to be essential.

Co-cultures of Sertoli cells and germ cells

Sertoli cells are the physiological nurse cells for germ cells; therefore, much effort has been devoted to the setting-up and development of Sertoli cell cultures. These efforts aimed to characterize Sertoli cells, with particular emphasis on the regulation of spermatogenesis and of the Sertoli cell endocrine function during both fetal (antimüllerian hormone) and postnatal stages (e.g., inhibin production).

A major methodological breakthrough occurred in 1975 when several laboratories developed reproducible protocols for the isolation and culture of Sertoli cells from immature and adult rat testes (Dorrington et al., 1975; Steinberger et al., 1975; Welsh and Wiebe, 1975). Improved protocols now make this possible on a routine basis. The key factors of these protocols are sequential enzymatic digestions and gravity sedimentation of Sertoli cell clusters, plus extensive washes (Skinner and Fritz, 1985; Toebosch et al., 1989). It is important to note that prepubertal animals are generally used as a source of cultured cells and allowed the development of culture systems for the study of spermatogenesis (for reviews, see Russell and Steinberger, 1989; Jégou, 1993).

Because Sertoli cells dedifferentiate when they are cultured and their preparation is tedious, several groups have attempted to establish immortalized Sertoli cell lines. A number of these cell lines are available and are listed in Table 1.1.

The concept of immortalizing Sertoli cells is obviously most interesting, but the cell lines generated only partially conserved the structural and molecular characteristics of the "real" Sertoli cells in situ, and only two cell lines, those used by Hofmann et al. (1992) and 15P-1 used by Rassoulzadegan et al. (1993), have been used to study in vitro spermatogenesis.

De Felici and Siracusa (1985) initially showed that PGC from 12.5 to 15.5 dpc mouse embryos adhere to Sertoli and follicular cells obtained from adult gonads. Since this time, much effort has been made to study PGC/gonocytes survival and proliferation in in vitro systems in which these germ cells are co-cultured on feeder cells. Maekawa and Nishimune (1991) developed a system based on the enzyme-dispersed mouse neonatal testes and demonstrated that germ cells, as well as supporting cells, incorporate [^{3}H]-thymidine and progress through the cell cycle in vitro with no apparent loss of viability after three days of culture. De Felici and Pesce (1994b) subsequently demonstrated that mouse PGC adhere to different cell monolayers, such as STO, TM4, COS, and F9 cells. They suggested that cell–cell interactions are mediated by multiple mechanisms involving Steel factor, c-Kit, carbohydrates, and possibly other unknown factors. The same study showed that (i) Steel factor and LIF prevent PGC death by suppressing apoptosis and are, therefore, survival factors; (ii) dibutyryl cAMP and forskolin, which are known to enhance intracellular levels of cAMP, stimulate PGC proliferation; and (iii) PACAP-27 and PACAP–28 may be physiological activators of adenylyl cyclase in PGC. Co-culture systems have shown that some factors are able, directly or via the co-cultured feeder cells, to modulate the number, proliferation, and/or survival of PGC and/or gonocytes. Such factors can increase (Steel factor: Godin et al., 1991; LIF: Resnick et al., 1992; fibroblast growth factor 2: Resnick et al., 1992, 1998; van Dissel-Emiliani et al., 1996) or decrease (TGF-β_1: Godin and Wylie, 1991) the number of germ cells.

Interestingly, primary cultures of PGC/gonocytes on suitable cell feeder layers, in particular adult or neonatal Sertoli cells, have permitted the survival of germ cells to be studied. They have also allowed cellular processes and structural features that resemble those observed i*n vivo* to be established. Gonocytes from 4 to 6 dpc adhere to an underlying neonatal Sertoli cell monolayer and survive for over seven days (Orth and Boehm, 1990). McGuinness and Orth (1992) demonstrated that gonocytes cultured with Sertoli cells can reinitiate mitosis, without added factors. Similarly, Resnick et al. (1992) showed that PGC can proliferate for up to seven days in a feeder-

Table 1.1. Characteristics of established Sertoli cell lines

Name	Permissive temperature	Species	Major Sertoli cell features	Origin	References
TM4[a]	No	Mouse	Transferrin, RBP, PA	Primary testis at 13 days postpartum	Mather, 1980
MSC-1	Yes	Mouse	Transferrin, SGP-2, β-inhibin, β-SGP-1, ABP	Transgenic	Peschon et al., 1992; McGuinness et al., 1994
S14-1	Yes	Mouse	Transferrin, SGP-2	Primary testicular cells	Boekelheide et al., 1993
15P-1 (unpure)	No	Mouse	Steel factor, WT1	Transgenic	Rassoulzadegan et al., 1993
ASC-17D	Yes	Rat	Transferrin, SGP-2	Sexually mature testis	Roberts et al., 1995
SK11 and SK49	No	Mouse	Steel factor, transferrin, SGP-2, α-inhibin, SF-1, GATA-1, RFSH	Transgenic	Walther et al., 1996
SMAT1	No	Mouse	AMH, SF-1	Transgenic	Dutertre et al., 1997
93RS2	Yes	Rat	Transferrin, SGP-2	Prepubertal testis	Jiang et al., 1997
SerW3	No	Rat	Transferrin	Primary testis at 17 days postpartum	Pognan et al., 1997
42 GPA9	No	Mouse	RFSH	Transgenic	Bourdon et al., 1998
MSC-1/RFSH	No	Mouse	RFSH	Transgenic	Eskola et al., 1998
TM4/ABP	No	Mouse	ABP	Original TM4 line	Ducray et al., 1998
45T-1	No	Mouse	Laminin	Transgenic	Szalay et al., 1999

Notes:

RBP, retinol-binding protein; PA, plasminogen activator; SGP, sulfated glycoprotein; WT1, Wilm's tumor 1; AMH, antimüllerian hormone; RFSH, follicle-stimulating hormone receptor; SF-1, steroidogenic factor 1; ABP, androgen-binding protein.

[a] This line is available through the American Type Culture Collection (ATCC; Rockville, MD, USA) and the European Collection of Animal Cell Cultures (ECACC; Valbonne, France).

dependent culture. Interestingly, the presence of an underlying laminin-containing matrix (matrigel) seems to be a prerequisite for the development of cellular extensions by the gonocytes (Orth and McGuinness, 1991). Desmosome-like attachments and gap junctions have also been observed in co-culture. The presence of gap junctions suggests that gonocytes become metabolically coupled to Sertoli cells (Orth and Boehm, 1990) and that diffusible factors can pass between gonocytes and Sertoli cells (Orth and McGuinness, 1991; van Dissel-Emiliani et al., 1993; Orth and Jester, 1995). Furthermore, electron microscopy has shown that quiescent gonocytes isolated from 18 dpc rats, co-cultured with 21–23 dpc Sertoli cells, establish numerous adhesion plaques between the two types of cell (van Dissel-Emiliani et al., 1993). Finally, PGC express several surface molecules that mediate adhesive interactions of PGC with the extracellular matrix, somatic cells and neighboring PGC; these include integrins, E-cadherin, tyrosine kinase receptor c-Kit, and specific types of oligosaccharide (De Felici, 2000; Di Carlo and De Felici, 2000).

Galdieri et al. (1984), Le Gac et al. (1984) and Le Magueresse et al. (1986) developed the first Sertoli–germ cell co-culture systems that used meiotic and postmeiotic germ cells. These authors used "pure" Sertoli cell preparations from prepubertal rat testes (20 dpp) and highly enriched populations of pachytene spermatocytes, early spermatids, and cytoplasts

from late spermatids to study the influence of germ cells on the morphology and secretory functions of Sertoli cells (Jégou, 1993; Jégou and Sharpe, 1993). However, this co-culture system was first used to demonstrate that rat pachytene spermatocytes can achieve the meiotic process in vitro in 1991 (Le Magueresse-Battistoni et al., 1991). In this study, germ cell maturation was monitored objectively by use of a purified population of pachytene spermatocytes. Flow cytometry was used to assess the appearance of a haploid peak and to analyze the spermatid-specific protamine-1 mRNA in co-culture extracts. It was demonstrated that under such experimental conditions a low proportion of spermatocytes (10%, mostly advanced in meiosis prophase) could enter meiosis and that, within the seven days the co-culture lasted, differentiation of spermatids could progress to step VII, which is consistent with the corresponding situation in vivo.

A different co-culture system was developed by the Rassoulzadegan and Cuzin group, who established testicular cell lines from transgenic mice expressing the polyoma virus (PyLT) protein in the testis (Paquis-Flucklinger et al., 1993). The 15P-1 cell line, which exhibits several features characteristic of Sertoli cells, was selected because it was claimed that this cell line supported meiotic and postmeiotic differentiation of germ cells collected from four-month-old mice transgenic for bacterial *LacZ* under the control of the haploid-specific protamine-1 promotor (Rassoulzadegan et al., 1993). This was one of the most astonishing studies ever published on in vitro spermatogenesis from a technical standpoint (the use of different transgenic mice, Sertoli cell lines, flow and image cytometry, light microscropy, thymidine labeling, and PCR) and in terms of the extraordinary results obtained. The 15P-1 cell line was claimed to support meiosis and full spermiogenesis up to the formation of a large number of spermatozoa (43-fold increase) in five days instead of the 35 days required in vivo. The 15P-1 line is not a pure Sertoli cell line as stated in Grandjean et al. (1997) and Rassoulzadegan et al. (1993), as it contains a significant germ cell component (Paquis-Flucklinger et al., 1993). However, our main criticism of this study is that a very crude mixture of germ cells was added to the 15P-1 cell line; this mixture contained various somatic elements and a large proportion of haploid cells, including spermatozoa. This created a "messy" background in which the appearance of newly generated haploid cells was largely blunted. This problem was not necessarily solved by the use of germ cells expressing the *LacZ* reporter gene, as non-specific staining can be observed in germ cells from normal mice when exposed to the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (M. O. Lienard, C. Pineau and B. Jégou, unpublished observations). This is most probably because of the high endogenous β-galactosidase activity in normal mouse testes (Cleutjens et al., 1997). The fact that Rassoulzadegan et al. (1993) observed β-galactosidase accumulation in the testes of 18-day-old mice, although it is generally observed that no or a very low number of spermatids normally exist at this age, and that no sperm cells appeared in the culture when no sperm cells were added (addition of a testicular cell suspension from immature mice), reinforce our conviction that the incredible germ cell proliferation seen in this study is probably largely artefactual. Finally, it is noteworthy that, to the best of our knowledge, seven years after this publication no other laboratory has published similar data using this cell line (or other cell lines), despite the extraordinary interest this new model generated. Despite intensive efforts (some of them in close collaboration with the authors), we were unable to reproduce the data published or to observe any germ cell differentiation when purified mice pachytene spermatocytes were used in the co-culture instead of the crude testicular mixture of cells. It is noteable that purified mice pachytene spermatocytes and early spermatids did not adhere well to the 15P-1 cell line, in contrast they adhered well to primary mice Sertoli cells (M. O. Lienard, C. Pineau and B. Jégou, unpublished observations).

Interestingly, when fresh pachytene spermatocytes were co-cultured with the immortalized somatic cells, including Sertoli cells, they were integrated tubule-like structures and some of them could survive for at least seven days (Hofmann et al., 1992).

In a more recent study, rat germ cell differentiation was carefully monitored. Purified germ cells, germ cell-specific complementary DNA probes, and 5-BrdU were co-cultured for 2 weeks with primary Sertoli cells from pubertal rat donors (20 dpp) in a bicameral chamber system (Weiss et al., 1997). These authors demonstrated that, despite massive germ cell death, a proportion of pachytene spermatocytes passed through the meiotic process and developed into early spermatids.

Conclusions

After 80 years of research on in vitro spermatogenesis, which has mobilized the most traditional and modern in vitro technologies (e.g., traditional culture of testicular explants; modern genetically modified testicular cells), and despite the crucial importance of the goals, little progress has been made in this domain. A general rule has arisen: the less the seminiferous tubule architecture is perturbed during tissue/cell preparation(s), the higher the chances are that a modest proportion of germ cells will pass through the different steps of in vitro spermatogenesis. This emphasizes the narrow limits of the technology available in this domain.

Nature resists the in vitro investigation of spermatogenesis because evolution has established an extraordinary interdependency of the cellular constituents of the testis. This interdependency is centered around a highly sophisticated network of anatomical and functional relationships between testicular cells, including the clonal arrangement of the different germ cell generations, the diverse structural and biochemical interconnections between the Sertoli cells themselves, and the connections between Sertoli cells and germ cells. This network is the evolutionary prerequisite for the production of an enormous and continous quantity of spermatozoa by the testes throughout the postpubertal life of mammals. It is estimated that an average human male produces 1500 to 2000 billion spermatozoa in his lifetime.

The most significant results of eight decades of research on in vitro spermatogenesis is the involvement of aspects of the endocrine system in the regulation of spermatogenesis and the determination of the nature of germ cell–Sertoli cell interactions.

There is undoubtedly still a margin for future improvement in this domain that can be used to improve the survival and maturation of germ cells. For example, the recent discovery that germ stem cells can survive for weeks in culture on a feeder layer opens major prospects (Shinohara et al., 2000). The development of this new technique will be invaluable to the scientists working on the germ cell molecular machinery and its control mechanisms. The most crucial objective is to develop further techniques for culturing or immortalizing germ stem cells. These in vitro systems would be useful for heuristic studies and would also allow stem cell multiplication for subsequent transplantation into sterile recipients. This technique would also be an advantage in zootechnology, in particular for the reproduction of high-value domestic animals and endangered mammalian species.

In humans this creates prospects for the treatment of male sterility. The gametes matured in vitro can be injected by germ cell repopulation of empty seminiferous tubules or used for ICSI. However, undertaking such microinjections at the current time would be irresponsible, as the risk of genetic abnormalities occurring in these cells is extremely high because they are immediately oxidized upon removal from their natural cryptic environment in situ, and because of the serious additional degenerative processes they are exposed to during isolation (necrosis and/or apoptosis). Consequently, there is presently no technique that can guarantee that the injected human cells are free from these problems.

Finally, the possibility of culturing spermatogonia automatically opens the way for their genetic manipulation and, therefore, for the generation of transgenic animals, which would be a major breakthrough. In humans, the possibility of genetically modifying germ cells could only be envisaged for the development of gene therapy if the present international ban on germ cell genetic manipulation was

lifted. It must still be open to question whether such a move would be desirable.

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